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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)
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RADEMACHER et al.) Art Unit: 1616
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Serial No. 10/009,629) Examiner: A. Pryor
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Filed: December 12, 2001)
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For: METHOD OF GENERATING PLANTS WITH AN INCREASED CONTENT OF FLAVONOIDS AND PHENOLIC CONSTITUENTS

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RESPONSE UNDER 37 CFR 1.116

In response to the Office action of January 7, 2003, applicants enclose a verified English translation fo the priority document No. 199 27 571.8. Applicants urge that the rejection under 35 U.S.C. 102(a) in view of Basak et al. has been overcome because this reference is not prior art with respect to the present application.

Please charge any shortage in fees due in connection with the filing of this paper, including Extension of Time fees to Deposit Account No. 11.0345. Please credit any excess fees to such deposit account.

Respectfully submitted,
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I, Margrit METHUEN Dipl.-Ing., Dr,

translator to RWS Group plc, of Europa House, Marsham Way, Gerrards Cross, Buckinghamshire, England declare;

1. That I am a resident of the United Kingdom of Great Britain and Northern Ireland.

2. That I am well acquainted with the German and English languages.

3. That the attached is, to the best of my knowledge and belief, a true translation into the English language of the accompanying copy of the specification filed with the application for a patent in Germany on 17 June 1999 under the number 199 27 571.8 and the official certificate attached hereto.

4. That I believe that all statements made herein of my own knowledge are true and that all statements made on information and belief are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application in the United States of America or any patent issuing thereon.

Margrit Methuen

For and on behalf of RWS Group plc

The 26th day of March 2003

FEDERAL REPUBLIC OF GERMANY

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Priority Certificate

for the filing of a Patent Application

File Reference: 199 27 571.8

Filing date: 17 June 1999

Applicant/Proprietor: BASF Aktiengesellschaft,
Ludwigshafen/DE

Title: Method of generating plants with an increased content of flavonoids and phenolic constituents

IPC: A 01 N 37/06

The attached documents are a correct and accurate reproduction of the original submission for this Application.

Munich, 06 July 2000

German Patent and Trademark Office

The President

pp

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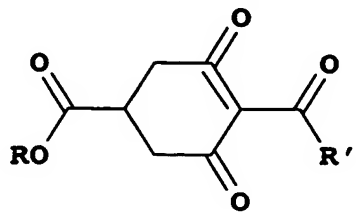
[signature]

Hiebinger

Method of generating plants with an increased content of flavonoids and phenolic constituents.

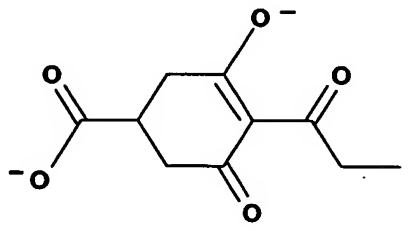
The present invention relates to a method of increasing the content of flavonoids and phenolic constituents in plants, wherein the plant [sic] are treated with growth-regulating acylcyclohexadiones [sic] of the formula I.



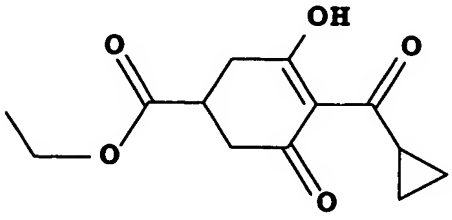
(I)

where R is, in particular, hydrogen, C_1-C_6-alkyl, C_1-C_6-haloalkyl, C_2-C_{10}-alkylthioalkyl or phenyl (substituted or unsubstituted) and R' is hydrogen, C1-C6-alkyl [sic], C3-C6-cycloalkyl [sic], benzyl (substituted or unsubstituted), phenylethyl, phenoxyethyl, 2-thienylmethyl, alkoxymethyl or alkylthiomethyl, and suitable salts of these compounds.

A method in which the increase are [sic] caused by treatment with acylcyclohexanediones such as prohexadione-calciumlcium (I) and/or trinexapac-ethyl (II) is especially preferred.



Ca^{++} (II)



(III)

The invention furthermore relates to the use of plants which have been treated by the method according to the invention with acylcyclohexadiones [sic] of the formula I, specifically prohexadione-calcium or with trinexapac-ethyl, or of parts of these plants or of products prepared with them (juices, infusions, extracts, fermentation products and fermentation residues) for the preparation of curative compositions,

health-promoting compositions or tonics for humans and animals, and of cosmetics.

The invention furthermore relates to compositions prepared by the methods according to the invention wherein the grapes of red grapevines are harvested and processed whose anthocyanin production has been prevented fully or partially by treatment with acylcyclohexanediones such as prohexadione-calcium or trinexapac-ethyl and which are therefore distinguished by a qualitatively and quantitatively increased content of flavonoids and other phenolic constituents.

A variety of phenolic substances (phenylpropanoids) are found in plants, for example caffeic acid, ferulic acid, chlorogenic acid, gallic acid, eugenol, lignans, coumarins, lignin, stilbenes (polydatin, resveratrol), flavonoids (flavones, catechines, flavanones, anthocyanidines, isoflavones), polymethoxylated flavones. Accordingly, phenols are also a general component in a large number of plant-derived foodstuffs and stimulants. Certain phenolic substances are of particular importance since, after ingestion together with the food, they may exert an antioxidant effect in the human or animal metabolism (Baum, B. O.; Perun, A. L. Antioxidant efficiency versus structure. *Soc. Plast. Engrs Trans* **2**: 250-257, (1962); Gardner, P.T.; McPhail, D.B.; Duthie, G.G. Electron spin resonance spectroscopic assessment of the antioxidant potential of infusions in aqueous and organic media. *J. Sci. Food Agric.* ***76:*** 257-262, (1997); Rice-Evans, C. A.; Miller, N. J.; Pananga, G. Structure-antioxidant activity relationship of flavonoids and phenolic acids. *Free Radic. Biol. Med.* **20**: 933-956, (1996); Salah, N.; Miller, N. J.; Paganga, G.; Tijburg, L.; Bolwell, G. P.; Rice-Evans, C. Polyphenolic flavonoids as scavenger of aqueous phase radicals and as chain-breaking antioxidants. *Arch Biochem Biophys* **322**: 339-346, (1995); Stryer, L. *Biochemistry* S. Francisco: Freeman, (1975); Vieira, O.; Escargueil-Blanc, I.; Meilhac, O.; Basile, J. P.; Laranjinha, J.; Almeida, L.; Salvayre, R.; Negre-Salvayre, A. Effect of dietary phenolic compounds on apoptosis of human cultured endothelial cells induced by oxidized LDL. *Br J Pharmacol* **123**: 565-573, (1998)). In addition, polyphenols have a multiplicity of effects on the cellular metabolism. Inter alia, signal transduction enzymes such as protein kinase C, tyrosine protein kinase and phosphatidylinositol 3-kinase are modulated (Agullo, G.; Gamet-payrastre, L.; Manenti, S.; Viala, C.; Remesy, C.; Chap, H.; Payrastre, B. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. *Biochem Pharmacol* **53** :1649-1657, (1997); Ferriola, P. C.; Cody,

V.; Middleton, E. Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure activity relationship. *Biochem Pharmacol* **38**: 1617-1624, (1989); Cushman, M.; Nagarathman, D.; Burg, D. L.; Geahlen, R. L. Synthesis and protein-tyrosine kinase inhibitory activity of flavonoids analogues. *J Meed Chem* **34**: 798-806, (1991); Hagiwara, M.; Inoue, S.; Tanaka, T.; Nunoki, K.; Ito, M.; Hidaka, H. Differential effects of flavonoids as inhibitors of tyrosine protein kinases and serine/threonin protein kinases. *Biochem Pharmacol* **37**: 2987-2992, (1988)), which downregulates inducible NO-synthase (Kobuchi, H.; Droy-Lefaix, M. T.; Christen, Y.; Packer, L. *Ginkgo biloba* extract (EGb761): inhibitory effect on nitric oxide production in the macrophage cell line RAW 264.7. *Biochem Pharmacol* **53**: 897-903, (1997)) and which regulates the gene expression of, for example, interleukins and adhesion molecules (ICAM-1, VCAM-1) (Kobuchi, H.; Droy-Lefaix, M. T.; Christen, Y.; Packer, L. *Ginkgo biloba* extract (EGb761): inhibitory effect on nitric oxide production in the macrophage cell line RAW 264.7. *Biochem Pharmacol* **53**:897-903, (1997); Wolle, J.; Hill, R. R.; Ferguson, E.; Devall, L. J.; Trivedi, B. K.; Newton, R. S.; Saxena, U. Selective inhibition of tumor necrosis factor- induced vascular cell adhesion molecule-1 gene expression by a novel flavonoid. Lack of effect on transcriptional factor NF-kB. *Atherioscler Thromb Vasc Biol* **16**: 1501-1508, (1996)). It is proven that these effects have a positive action for preventing cardiovascular diseases, diabetes, various kinds of tumors and other chronic diseases (Bertuglia, S.; Malandrino, S.; Colantuoni, A. Effects of the natural flavonoid delphinidin on diabetic microangiopathy. *Arznei-Forsch/Drug Res 45: 481-485*, (1995); Griffiths, K.; Adlercreutz, H.; Boyle, P.; Denis, L.; Nicholson, R.I.; Morton, M.S. *Nutrition and Cancer* Oxford: Isis Medical Media, (1996); Hertog, M. G. L.; Fesrens, E. J. M.; Hollman, P. C. K.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. *The Lancet* **342**: 1007-1011, (1993); Kapiotis, S.; Hermann, M.; Held, I.; Seelos, C.; Ehringer, H.; Gmeiner, B. M. Genistein, the dietary-derived angiogenesis inhibitor, prevents LDL oxidation and protects endothelial cells from damage by atherogenic LDL. *Arterioscler Thromb Vasc Biol* **17**: 2868-74, (1997); Stampfer, M. J.; Hennekens, C. H.; Manson, J. E.; Colditz, G. A.; Rosner, B.; Willet, W. C. Vitamin E consumption and the risk of coronary disease in women. *New Engl J Med* **328** :1444-1449, (1993); Tijburg, L. B. M.; Mattern, T.; Folts, J. D.; Weisgerber, U. M.; Katan, M. B. Tea flavonoids and cardiovascular diseases: a review. *Crit Rev Food Sci Nutr* **37**: 771-785, (1997); Kirk, E. A.; Sutherland, P.; Wang, S. A.; Chait, A.; LeBoeuf, R. C. Dietary isoflavones reduce plasma cholesterol and

atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice. *J Nutr* **128**: 954-9, (1998)). A series of curative compositions, health-promoting compositions or tonics whose action is based on their content of phenolic substances is therefore already being obtained from suitable plants (Gerritsen, M. E.; Carley, W. W.; Ranges, G. E.; Shen, C. P.; Phan, S. A.; Ligon, G. F.; Perry, C. A. Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. *Am J Pathol* **147**: 278-292, (1995); Lin, J. K.; Chen, Y. C.; Huang, Y. T.; Lin-Shiau, S. Y. Suppression of protein kinase C and nuclear oncogene expression as possible molecular mechanisms of cancer chemoprevention by apigenin and curcumin. *J Cell Biochem Suppl* **28-29**:39-48, 1997; Zi, X.; Mukhtar, H.; Agarval, R. Novel cancer chemopreventive effects of a flavonoid antioxidant silymarin: inhibition of mRNA expression of an endogenous tumor promoter TNF alpha. *Biochem Biophys Res Comm* **239**:334-339, 1997). It is also known that certain plant-derived foodstuffs or stimulants prepared from them have a positive effect on various diseases. Resveratrol, which occurs in white wine, but in particular in red wine (in addition to other components), for example, is active against cardiovascular diseases and cancer (Gehm, B.D.; McAndrews, J.M.; Chien, P.-Y.; Jameson, J.L. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for estrogen receptor. *Proc Natl Acad Sci USA* ***94***: 14138-14143, (1997); Jang, M.; Cai, L.; Udeani, G.O.; Slowing, K.V.; Thomas, C.F.; Beecher, C.W.W.; Fong, H.H.S; Farnsworth, N.R.; Kinghorn, A.D.; Mehtha, R.G.; Moon, R.C., Pezzuto, J.M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* ***275***: 218-220, (1997)). A similar effect is also found in substances such as catechin, epicatechin-3-gallate, epigallocatechin and epigallocatechin-3-gallate, which are found in the leaves of tea (*Camellia sinensis*). Beverages, in particular those made with unfermented tea leaves (green tea), are beneficial for health (Hu, G.; Han, C.; Chen, J. Inhibition of oncogene expression by green tea and (-)-epigallocatechin gallate in mice. *Nutr Cancer* **24**: 203-209; (1995); Scholz, E; Bertram, B. *Camellia sinensis* (L.) O. Kuntze. Der Teestrauch [the tea shrub]. *Z. Phytotherapie* ***17***: *235-250*, (1995); Yu, R.; Jiao, J. J.; Duh, J. L.; Gudehithlu, K.; Tan, T. H.; Kong, A. N. Activation of mitogen-activated protein kinases by green tea polyphenols: potential signaling pathways in the regulation of antioxidant responsive elements-mediated phase II enzyme gene expression. *Carcinigenesis* **18**: 451-456, (1997); Jankun, J.; Selman, S.H.; Swiercz, R. Why drinking green tea could prevent cancer. *Nature* ***387***: *561*, (1997)). In addition, polymethoxylated flavones from citrus fruits also have a potential antitumor action (Chem, J.;

Montanari, A.M.; Widmer, W.W. Two new polymethoxylated flavones, a class of compounds with potential anticancer activity, isolated from cold pressed dancy tangerine peel oil solids. *J Agric Food Chem 45: 364-368*, (1997)).

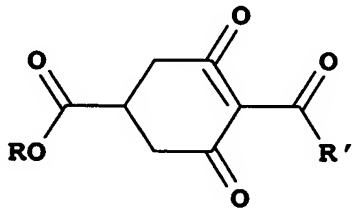
Acylcyclohexanediones such as prohexadione-calcium and trinexapac-ethyl (earlier name: cimectacarb) are employed as bioregulators for inhibiting longitudinal growth in plants. Their bioregulatory action is based on their blockage of the biosynthesis of gibberellins, which promote longitudinal growth. Owing to their structural relationship with 2-oxoglutaric acid, they inhibit certain dioxygenases which require 2-oxoglutaric acid as co-substrate (Rademacher, W, Biochemical effects of plant growth retardants, in: Plant Biochemical Regulators, Gausman, HW (ed.), Marcel Dekker, Inc., New York, pp. 169-200 (1991)). It is known that such compounds also engage in the phenol metabolism and can therefore cause inhibition of anthocyanin production in various kinds of plants (Rademacher, W et al., The mode of action of acylcyclohexanediones - a new type of growth retardant, in: Progress in Plant Growth Regulation, Karssen, CM, van Loon, LC, Vreugdenhil, D (eds.), Kluwer Academic Publishers, Dordrecht (1992)). Such effects on the balance of phenolic constituents are given as the cause of the side effect of prohexadione-calcium against fire blight (Rademacher, W et al., prohexadione-Ca - a new plant growth regulator for apple with interesting biochemical features, Poster presented at the 25th Annual Meeting of the Plant Growth Regulation Society of America, July 7-10, 1998, Chicago). A. Lux-Endrich (PhD thesis at the Technical University Munich at Weihenstephan, 1998) found during her studies into the mechanism of action of prohexadione-calcium against fire blight that, in apple tissue cultures, prohexadione-calcium results in the content of phenolic substances being increased several times and that a series of phenols is found which is otherwise not present. It was also found during this study that exposure to prohexadione-calcium leads to relatively large amounts of luteoliflavan and eriodyctiol [sic] in the shoot tissue of apples. Luteoliflavan does normally not occur in apple tissue, and eriodyctiol [sic] occurs only in small amounts as an intermediate in the flavonoid metabolism. However, the expected flavonoids catechin and cyanidin were not detectable in the treated tissue, or found in considerably reduced amounts only (S. Römmelt et al., paper presented at the 8th International Workshop on Fire Blight, Kusadasi, Turkey, October 12-15, 1998).

It can be considered as proven that prohexadione-calcium, trinexapac-ethyl and other acylcyclohexanediones inhibit 2-oxoglutaric-acid-dependent hydroxylases which are of importance

in the metabolism of phenolic substances. These hydroxylases are primarily chalcone synthetase (CHS) and flavanone 3-hydroxylase (F3H) (W. Heller and G. Forkmann, Biosynthesis, in: The Flavonoids, Harborne, JB (ed.), Chapman and Hall, New York, 1988). However, it cannot be excluded that acylcyclohexanediones also inhibit other 2-oxoglutaric-acid-dependent hydroxylases which are as yet unknown. Furthermore, it should be obvious that lack of catechin, cyanidin or other end products of flavonoid synthesis is registered by the plant and that the activity of the key enzyme phenylalanine ammonium-lyase (PAL) is increased by a feedback mechanism. However, since CHS and F3H are still being inhibited, these flavoniod [sic] end products cannot be formed, and the result is an increased production of luteoliflavan, eriodyctiol [sic] and other phenols (Figure 1).

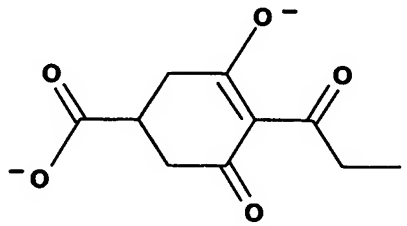
It is an object of the present invention to provide an economic, simple method for increasing the content of flavonoids and phenolic compounds in plants and to improve their health-promoting properties.

We have found that this object is achieved, surprisingly, by treating the plants with the growth-regulating compounds from the group of the acylcyclohexanediones (I)



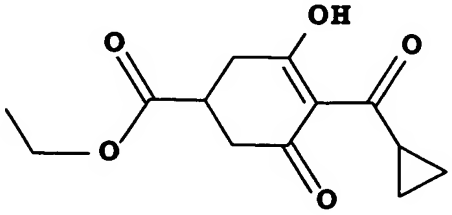
(I)

in particular with the compounds prohexadione-calcium (II)



Ca^{++} (II)

and trihexapac-ethyl (III)



(III).

Treatment of the plants with the acylcyclohexadiones [sic] of the formula (I), prohexadione-calcium (II) and trinexapac-ethyl (III) allows the flavonoids eriodictyol, proanthocyanidines, which are substituted on the C-atom 3 by hydrogen, for example luteoforol, luteoliflavan, apigeniflavan and tricetiflavan, and homogeneous and heterogeneous oligomers and polymers of the abovementioned, structurally related, substances to be formed in greater quantities.

Increased concentrations of the phenols hydroxycinnamic acid (p-coumaric acid, ferulic acid, sinapic acid,) salicylic acid or umbelliferone, including the homogeneous and heterogeneous oligomers and polymers formed with them, can be identified after the compounds acylcyclohexadiones [sic] of the formula (I), prohexadione-calcium (II) and trihexapac-ethyl (III) have been applied to plants.

The concentration of the glycosides of the flavonoids, of the phenolic compounds, of the chalcones and of the stilbenes in the plants is also increased by treating the plants with the acylcyclohexadiones [sic] of the formula I, prohexadione-calcium (II) and trinexapac-ethyl (III).

Also, prohexadione-calcium, trinexapac-ethyl and related compounds engage in other metabolic reactions where, as yet, it is only possible to assume that 2-oxoglutarate-dependent dioxygenases are involved.

A further additional positive effect when obtaining preparations from higher plants with an improved curative, health-promoting or tonifying action is that, owing to the growth-regulatory action of prohexadione-calcium, trinexapac-ethyl or related acylcyclohexanediones, a concentration effect of the relevant constituents results in the biological material.

The method according to the invention for increasing the content of flavonoids and phenolic constituents by treating the plants with compounds from the group of the acylcyclohexadiones [sic] of the formula I, specifically prohexadione-calcium or trinexapac-ethyl, can be applied successfully to the following plants, but it is also possible successfully to treat plants which are not mentioned: grapevines, cherries, plums, sloes, blueberries, strawberries, citrus fruit (such as oranges, grapefruit), pawpaw, red cabbage, broccoli, cauliflower, kale, carrots, parsley, celery/celeriac, onions, garlic, tea, coffee, cacao, maté, hops, soya, oilseed rape, oats, wheat, rye, Aronia melanocarpa and Ginkgo biloba acts [sic].

Plants which have been treated with compounds from the group of the acylcyclohexadiones [sic], specifically prohexadione-calcium or trihexapac-ethyl in order to increase the content of flavonoids and phenolic compounds, or of [sic] parts of these plants or products prepared from them (juices, infusions, extracts, fermentation products and fermentation residues) can be used for preparation of curative compositions, health-promoting compositions or tonics for humans and animals, and of cosmetics.

It is also possible to prepare, from the plants which have been treated in accordance with the invention, compositions wherein grapes of red grapevines are harvested and processed whose anthocyanin production has been prevented fully or partially by treatment with acylcyclohexadiones [sic] such as prohexadione-calcium or trinexapac-ethyl and which are therefore distinguished by a qualitatively and quantitatively increased content of flavonoids and other phenolic constituents.

Surprisingly, it has been found that under the influence of plants treated with acylcyclohexanediones of the formula I, prohexadione-Ca or trihexapac-ethyl or of parts of these plants or products prepared from them (infusions, extracts, fermentation products, juices and the like)

(1) the antioxidative capacity *in vitro* (electron spin resonance (ESR), LDL oxidation, total antioxidant capacity, NO scavenging) is improved;

(2) a modulating effect on enzymes, especially signal transduction enzymes (protein kinase C, tyrosin protein kinase, phosphatidylinositol 3-kinase) is observed;

(3) a modulation of redox-sensitive transcriptional factors (NF-kB, AP-1) in endothelial cells, lymphocytes and smooth muscle cells is induced;

(4) the regulation of gene expression of target genes which are involved in the pathogenesis of inflammatory diseases (cytokines IL-1 and IL-8, *macrophage chemoattractant protein* 1 (MCP-1), adhesion factors ICAM-1 and VCAM-1) is modulated;

(5) an antiaggregatory action is induced;

(6) the cholesterol synthesis in hepatocytes is inhibited;

(7) antiproliferative/antineoplastic effects are observed.

Example 1

Increase of the eriodictyol and luteoliflavan content in young apple leaves following treatment with prohexadione-calcium.

Apple plants cv. "Weirouge" were grown under controlled-environment conditions and treated to runoff point with 250 ppm prohexadione-calcium (formulated as BAS 125 10 W = wettable granules, content 10%). At various points in time after the treatment, the youngest fully developed leaf was harvested from each individual shoot. The freeze-dried leaves which had been ground using a pestle and mortar were extracted with methanol. Flavonoids and related compounds in the concentrated extract were analyzed by HPLC. Separation was performed on Hypersil ODS (particle size 3 U [sic]) on a 250 x 4 mm column. Elution was carried out at a flow rate of 0.5 ml per minute, and mixtures of formic acid (5% in water) and methanol, increased stepwise from a ratio of 95 : 5 to 10 : 90 (v/v) were used. Phenolic acids and flavonols were detected at 280 nm. Flavan-3-ols were determined by post-column derivatization with p-dimethylaminocinnamaldehyde at 640 nm. For methodological details, see Treutter et al. (1994), Journal of Chromatography A 667, 290 - 297.

The result is shown in the table which follows.

Leaves treated with prohexadione-calcium show a markedly increased eriodictyol concentration after 12 and 21 days.

Treatment	Eriodictyol [g/kg dry matter]		Luteoliflavan [g/kg dry matter]	
	12 days after treatment	21 days after treatment	12 days after treatment	21 days after treatment.
Control	0	1	0	70
250 ppm prohexadione-calcium	17	27	0	34

Example 2

Preparation of sample materials from treated and untreated Dornfelder grapes

Vines cv. "Dornfelder" were treated twice at different points in time with the formulation BAS 125 10W, which contains prohexadione-calcium. 1000 g of prohexadione-calcium in 1000 l of spray mixture were applied per ha per treatment.

The 1st application was carried out at developmental stage 73 before the berries developed their color, and the 2nd application 10 days thereafter.

When harvested, the untreated and treated grapes showed a similar degree of ripeness. Untreated control: 69°C [sic] Oechsle, acid: 7.3 g/l; treated control: 67° Oechsle, acid: 7.4 g/l.

Pigmentation was less pronounced in the treated grapes. As regards taste, no difference was observed.

The grapes were made into red wine by customary methods, i.e. the must was left to stand on the pulp for a prolonged period to improve pigment extraction.

After the wine which was free from cloudiness had been freeze-dried, approx. 2.5 g of a syrupy residue was obtained from 100 ml of untreated wine and approx. 2.1 g of syrupy residue from the wine from those vines which had been treated with prohexadione-calcium.

Example 3

Inhibition of cholesterol biosynthesis in primary rat hepatocyte cultures by prohexadione-calcium treated Dornfelder wine.

Preparation of the stock solutions

A quantity of the lyophilisate of the untreated and treated Dornfelder wines of between 10 and 20 mg was weighed exactly and treated with such an amount of DMSO that a stock solution of 10 mM total flavonoids resulted. These stock solutions were used for preparing dilutions in the culture medium immediately prior to the beginning of the test. The dilutions were done in 10-fold dilution steps of between 10^{-4} and 10^{-8} M.

Preparation of the hepatocyte cultures

Primary hepatocytes were obtained from the livers of Spraque-Dawley [sic] rats (240-290 g) by means of collagenase perfusion (Gebhardt et al., Arzneimittel-Forschung/Drug Res. 41: 800 – 804 (1991) 1990). They were cultured in collagen-coated

Petri dishes (6-well plates, Greiner, Nürtingen) at a cell density of 125,000 cells/cm^2 in Williams medium E supplemented with 10% calf serum. More detailed information, in particular on the culture medium, are found in Gebhardt et al., Cell Biol. Toxicol. 6: 369 – 372 (1990) and Mewes et al., Cancer Res. 53: 5135 – 5142 (1993). After 2 h, the cultures were transferred to serum-free medium supplemented with 0.1 µM insulin. After a further 20 h, they were employed in the experiments. The test substances were each tested in three independent cultures of 2-3 rats.

Incubation of the liver cell cultures with the test substances

To demonstrate that cholesterol biosynthesis is influenced by the test substances, the hepatocyte cultures were maintained for 22 h in total. Then, they were incubated for 2 h in serum-free Williams medium E supplemented with ^{14}C-acetate (tracer quantities only) with the test substances at the concentrations indicated. Each test series included a control. The methodology is described in detail by Gebhardt (1991) and Gebhardt, Lipids 28: 613 – 619 (1993). The tracer quantities of ^{14}C-acetate exchange rapidly with the intracellular acetyl-CoA pool and therefore allow the incorporation of ^{14}C-acetate into the sterol fraction, > 90% of which consists of cholesterol, to be detected without interference (Gebhardt, 1993).

Analysis of the effect on cholesterol biosynthesis

The incorporation of ^{14}C-acetate into the sterol fraction (non-hydrolyzable lipids) was measured using the method of Gebhardt (1991). If the extraction is carried out by means of Extrelut® columns (Merck, Darmstadt), over 95% of the ^{14}C-acetate (and other low-molecular-weight metabolites formed therefrom in minor quantities) is removed. This test can provide comparative information on the relative synthesis rate of cholesterol and precursor sterols under the influence of test substances (Gebhardt, 1993).

Representation of the results, and statistics

The figures show the measurement data as mean values ± SD of the individual experiments after standardization based on the control values (= 100 %). Student's t-Test was employed for the statistical assessment; significant deviations with p-values of <0.02 were marked by (*).

Visual and microbial quality checks of the hepatocyte cultures

Before and after the test incubation, all cultures used were checked visually under the microscope for contamination with microorganisms and for the integrity of the cell monolayer. In none of the samples was a noticeable change in cell morphology observed (in particular at the higher concentrations). This largely rules out the possiblity that the test results were influenced by cytotoxic effects of the test substances.

The sterility tests, which were carried out routinely in all cultures, did not suggest any contamination with microorganisms.

Results

The untreated Dornfelder wine showed no effects whatsoever on cholesterol biosynthesis. In contrast, the cholesterol synthesis was inhibited significantly by samples of wine which originated from prohexadione-calcium-treated vines. At a concentration of 10^{-5} M, the inhibitory effect was approx. 60% and at 10^{-4} M almost 100%.